CHAPTER 17

Metal Relationships of Earthworms

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I. GENERAL INTRODUCTION

Earthworms are relatively large, soft-bodied detritivores: they usually feed on decomposing plant materials, but may also graze on roots and consume seeds, algae, fungi, and protozoa.[1] Despite the surprisingly low taxonomic and morphological variability within such a well-established and functionally important animal group, earthworms can be subdivided into at least three ecophysiological categories:[2]

1. Epigeic Species — relatively small, usually pigmented, litter-inhabiting species;
2. Endogeic species — small or large, weakly pigmented worms living in horizontal burrows in the organomineral soil horizon; and
3. Anecic species — large worms living in deep vertical burrows that may also feed on the surface.

Other schemes of dividing earthworms have been adopted which complement rather than contradict the scheme proposed by Bouche.[2] For example, some earthworm species have active, mineralizing, calciferous glands that appear to be associated with the ability to tolerate a fairly wide soil pH range; other species have inactive or nonmineralizing glands.[3,4] Some species enter a discrete resting state, 'diapause', during periods of adverse climatic conditions; other species remain active or experience spells of reduced activity, 'quiescence', during certain periods of the year.[5] Earthworms are in fairly intimate contact with litter and soil, although they may be much more discriminate consumers than is often appreciated.[6] It has been convincingly shown that earthworms do accumulate several different metals from soils contaminated from diverse anthropogenic sources,[7,8] with tissue concentrations often exceeding those in organisms occupying higher trophic levels and inhabiting the same polluted location.[9] While these observations are consistent with those made on oligochaetes in freshwater food chains,[10] some caution needs to be exercised in the interpretation of these findings.

Terrestrial organisms such as geophilid centipedes (Myriapoda; Chilopoda) and slugs (Mollusca; Gastropoda; Pulmonata) spend relatively large proportions of their lives beneath the soil surface,[11] and yet they do not accumulate metals to the same extent as earthworms. A subterranean lifestyle *per se* does not lead to high accumulated metal concentrations, even in animals with soft, highly permeable integuments. Why, therefore, can earthworms accumulate high metal burdens? Answering this deceptively simple question is difficult, but it appears that it reflects the detritivorous habit, itself promoted by a resident gut microflora and the functional anatomy of the oligochaete alimentary system. This hypothesis can probably be extended to explain why terrestrial isopods are also prodigious metal accumulators.[9,12,13]

Attempting to explain metal accumulation goes to the crux of the issue of exposure. Exposure is not simply consumption. Metal accumulators must not

only consume metal-contaminated materials, either as selected dietary constituents, or as ingesta indiscriminately consumed during burrowing; the released metals must also be rendered available for transport across the absorptive epithelia. (Of course, the existence of these processes does not preclude the possibility that a proportion of the body burden derives from metal transport across integumentary and/or respiratory surfaces.) Finally, the metal accumulator must possess the capacity to sequester metals within its cells and tissues, either by binding to existing or induced ligands.

Overgeneralizations may conceal important information. For example:

1. Tissue metal accumulation is the net effect of uptake and excretion. An organism is a nonaccumulator for one of several reasons:
   - it is not exposed, in either the ecological or physiological senses, to the given metal;
   - it is exposed, but effectively excludes the metal by blocking access to transepithelial pathways;
   - it has a highly discriminating uptake pathway(s);
   - it has a high excretory rate;
   - it has a high uptake and low excretory rates, but poor intracellular sequestration/detoxification capacity, so that low body burdens are highly debilitating or lethal.
2. Accumulators may not accumulate all metals with comparable efficiencies. This is a principle that applies to both essential and nonessential metals. Earthworms and isopods, for example, are both metal accumulating groups,[9] but isopods are very avid Cu accumulators,[12] while worms do not accumulate high Cu concentrations even from relatively heavily contaminated soils.[13-15]
3. Even nonaccumulators can accumulate high concentrations of certain essential metals. For example, centipedes do not assimilate Pb; but even under noncontaminated conditions they contain high Zn burdens, the large proportion of which is restricted to a subcuticular tissue.[16]

Metal relationships, including the impact of exposure to toxic metals, can be examined at several different levels of functional organization, namely: ecological, physiological, cellular, and molecular. The present paper is confined to a brief review of recent findings on the ecological and cellular relationships between earthworms and certain essential (Zn) and nonessential (Pb, Cd) metals. We have two modest heuristic objectives: (1) to highlight information that may contribute to the establishment of general principles regarding metal accumulation, compartmentation, and detoxification by these large terrestrial detritivores; and (2) to draw attention to areas where further information is required.

II. EARTHWORM AND MONITORING SOIL METAL POLLUTION

Aquatic organisms have been used relatively extensively compared with terrestrial species for monitoring the relative degrees of pollution at different

sites. There appear to be two pragmatic motivations for their use: (1) efforts to safeguard human health by measuring accumulated metal concentrations in key food organisms, and subsequently comparing the observed data with standards established by toxicity tests; and (2) detecting spatial and temporal trends in metal contamination, so that emission control practices can be mobilized at the source(s). It is pertinent to ask why it is deemed desirable, if not necessary, to measure pollutants in living tissues rather than directly in an abiotic component of the study site(s).

The general concept of measuring the tissue concentrations of metals in a selected species to yield information concerning 'bioavailable pollution fractions' in a given habitat is theoretically attractive, but is fraught with practical difficulties. The fact that bioavailability data are species specific leads to interpretative problems. Differences have been recorded in the concentrations of metals accumulated by closely related species of slugs[9,17,18] and isopods[9,19-21] living, respectively, in the same contaminated habitats. In the case of isopods, the observed differences between the species *Porcellio scaber* and *Oniscus asellus* are attributed largely to differences in the turnover of certain metal-accumulating cells in their hepatopancreases.[19] It is not entirely unexpected that significant differences have also been found in the tissue metal concentrations of ecophysiologically different earthworm species: the endogeic species, *Apporrectodea caliginosa* and *Octolasion cyaneum*, accumulate higher in Pb and Zn concentrations than the epigeic species, *Lumbricus rubellus*.[22-25] However, detailed analyses of the metal concentrations in the crop contents, feces, and tissues of epigeic and endogeic species sampled from the same metalliferous site have failed to identify the basis of the observed species differences (Figure 1), but concluded that there are a number of interactive, mainly biotic, determinants.[24] Even less easy to explain are the striking differences that occur between two epigeic earthworms, *L. rubellus* and *Dendrodrilus rubidus*,[26-28] where the smaller *D. rubidus* accumulates higher concentrations of Pb (Figure 2) and Cd, but lower concentrations of Zn and Ca than *L. rubellus*.[28] Clearly the 'bioavailable metal fraction' estimated by analyzing tissue concentrations is not equivalent or analogous to any 'soil extractable fraction' estimated by standard physicochemical procedures. Because the measured accumulated metal concentrations in the tissues of a given biomonitoring organism are not absolute measures of either 'bioavailability' per se, or of metal 'speciation' within a soil, it is only valid to use such data as indices for comparing the relative and potential bioavailabilities of specific metals between different sites.

Having defined the objectives of the biomonitoring exercise and selected the biomonitoring species, other fundamental issues must be addressed. Unambiguous biomonitoring, according to Phillips,[29] can best be achieved if there is a relatively simple statistical relationship (i.e., linear relationship) between metal concentrations in the biomonitoring organism and in a relevant abiotic component of its environment. Even if this proposal were acceptable, a number of factors can detract from such a relationship, occasionally in unpredictable ways. Take, for example, the earthworm species *A. caliginosa* and *L. rubellus*. *A. caliginosa*,

but not *L. rubellus*, enters a distinctive resting state during dry spells in the temperate summer. During diapause, the tissue concentrations of Cd and Zn are significantly lower than in 'active' *A. caliginosa*, while Pb concentrations are significantly elevated during diapause.[25] We saw earlier that *A. caliginosa* is a more efficient accumulator of metals than *L. rubellus*, a fact that recommends it for biomonitoring purposes. However, the relationship between the metal concentrations in the tissues of *A. caliginosa* and in the soil that it inhabits is subject to change dictated by local climatic, and perhaps edaphic, conditions.

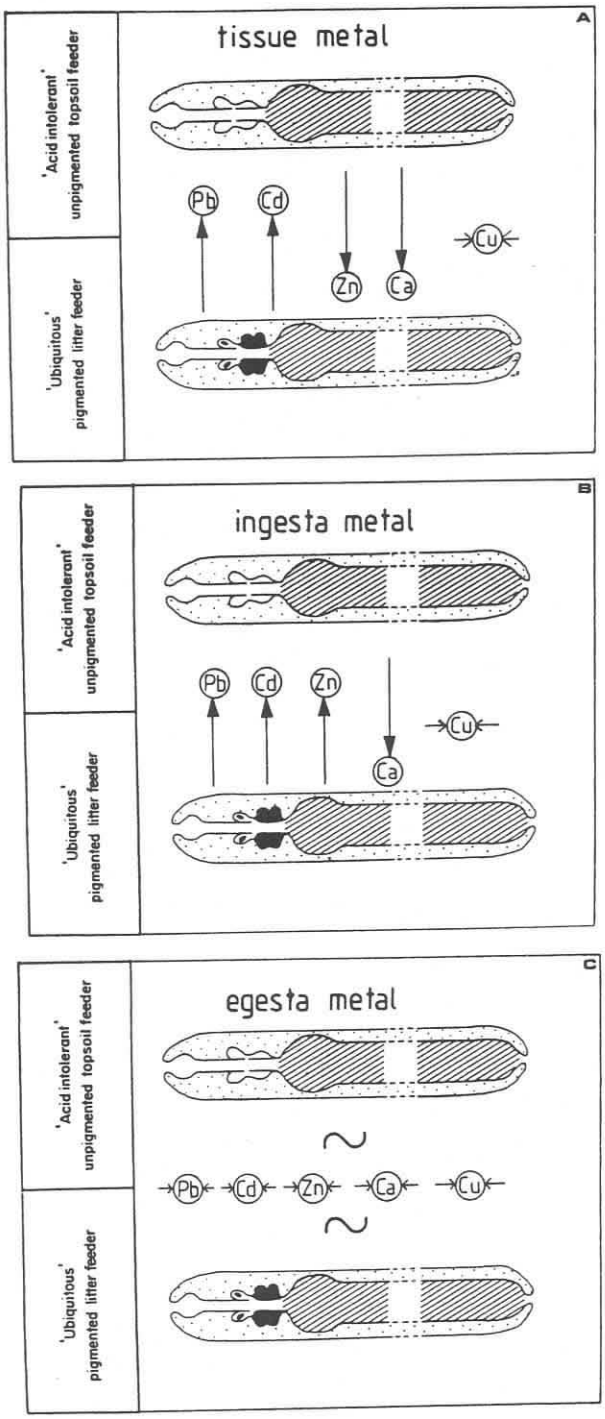
A linear relationship between metal concentrations in earthworm tissues and in soil suggests that the same information concerning relative pollution amounts at different sites could probably be derived more readily by the direct analysis of soils. However, the recognition of 'outliers', that deviate from the linear relationship, can reveal important information that integrates in space and time the interactive influences of abiotic and biotic factors.[30,31]

Two 'outlier' populations can be identified in the data plotted in Figure 3, each showing nonconformity for a different metal: (1) the Pb concentration in *L. rubellus* sampled from site "g" exceeded the prediction of the regression analysis, such that the concentration factor for this population was 3.9, compared with values well below unity for all other populations; and (2) the Cd concentrations in worms from site "f" were well below statistical prediction such that, of all the populations analyzed, this was the only one yielding a concentration factor below unity for this particular metal. It is of more than passing interest that site "g" soil is both acidic and relatively Ca poor, especially since it has been shown that both edaphic factors exert strong codeterminant influences on the availability of Pb to earthworms.[32-35] Moreover, the site "f" soil was notable because it had a Zn concentration (= 150,000 μg/g dry weight) about twice that of the next most contaminated soil, and it also had by far the highest organic matter content (loss on ignition = 35%). One or both of these factors may render the Cd at site "f" relatively unavailable. Certainly, the immobilizing influence of soil macromolecules is well known.[32,34]

'Outlier' populations could be explained not only by the interaction of edaphic synergists and antagonists, but also by the existence of intrinsic factors whereby earthworm ecotypes have evolved the capacity to either exclude or hyperaccumulate certain metals, due to the heavy selection pressures extant in their polluted environments. If such local adaptations are shown to exist, then it may be prudent to biomonitor by exposing genotypically uniform test organisms to the soils to be tested under field-enclosure or laboratory conditions.[36,37]

III. EFFECTS OF METALS ON EARTHWORMS IN THE FIELD: METAL INTERACTIONS ON A BIOCHEMICAL LEVEL OF ORGANIZATION

Earthworms are used extensively to evaluate the toxicities of xenobiotics by standardized acute toxicity tests.[38] A number of behavioral, reproductive, bio-


A
tissue metal
'Acid intolerant' unpigmented topsoil feeder
'Ubiquitous' pigmented litter feeder
Pb
Cd
Zn
Ca
Cu
B
ingesta metal
'Acid intolerant' unpigmented topsoil feeder
'Ubiquitous' pigmented litter feeder
Pb
Cd
Zn
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C
egesta metal
'Acid intolerant' unpigmented topsoil feeder
'Ubiquitous' pigmented litter feeder
Pb
Cd
Zn
Ca
Cu

FIGURE 1. Schematic diagrams summarizing analytical data on the metal concentrations in whole-worm tissue, ingesta (crop contents), and egesta (feces), of two very broad ecophysiological categories of earthworms sampled from five 'stations' at a disused Pb/Zn mine (Llantrisant: O.S. Map Ref. = ST 048822). All worms were analyzed individually; for smaller species, crop contents, and feces, respectively, from more than one worm were pooled to provide a sample. The 'ubiquitous' ecophysiological group includes *L. rubellus* (epigeic) and *L. terrestris* (anecic), both litter feeders if not litter inhabitors, and both possessing active mineralizing calciferous glands. The 'acid intolerant' group includes *A. caliginosa* (endogeic) and *A. longa* (anecic), both apparently consuming more mineralized materials, and both possessing inactive nonmineralizing calciferous glands. Upward pointing (↑) arrows indicate that the 'acid intolerant' species have significantly higher concentrations of a given metal; downward pointing (↓) arrows indicate that the 'ubiquitous' species have the higher concentrations; horizontal (→ ←) arrows indicate that there are no significant differences between the species groups. (These diagrams were constructed from data presented in tabular form by Morgan, J. E. and Morgan, A. J., *Soil Biol. Biochem.*, in press. With permission.)

chemical, and immunological performance indicators have also been developed for evaluating the sublethal toxicities of chemicals, including metals, to earthworms chronically exposed under laboratory and field conditions.[39-41] However, very few studies have been conducted to examine the effects of toxic metals on earthworms inhabiting heavily contaminated metalliferous soils. Such populations are not only chronically exposed to complex metal mixtures, and diverse edaphic factors, but have also had the opportunity of evolving over periods that could accommodate several tens, if not hundreds, of earthworm generations.

In earthworms, very high proportions of the total accumulated body burdens of Pb, Cd, and Zn are confined to the posterior alimentary tissues,[15] with the chloragogenous tissue separating the absorptive intestinal epithelium from the coelom being a major metal depository (see Figure 4).[26,27,42-44] Although the morphology of individual chloragocytes changes significantly due to the accumulation of toxic metals (see Figure 5),[26,42,45] these changes have not been accurately described nor measured morphometrically. Furthermore, it is not known whether the cellular effects are a reflection of an increased turnover of intracellular metal-sequestering organelles and/or the direct toxic effects of the extraneous metals. However, it appears that earthworm chloragocytes respond to Pb accumulation in a way that is analogous to the response of isopod 'B' cells[46] (see also Chapter 18). Lead accumulation reduces energy stores, in the form of glycogen, in the earthworm cells (Figure 5).[26,45] But the direct effects of toxic metals on the biochemical pathways involved in energy storage should not be dismissed.[47]

It is well known that low concentrations of Pb inhibit hemoglobin biosynthesis in vertebrates.[48] The metal can interfere with several steps in the haem

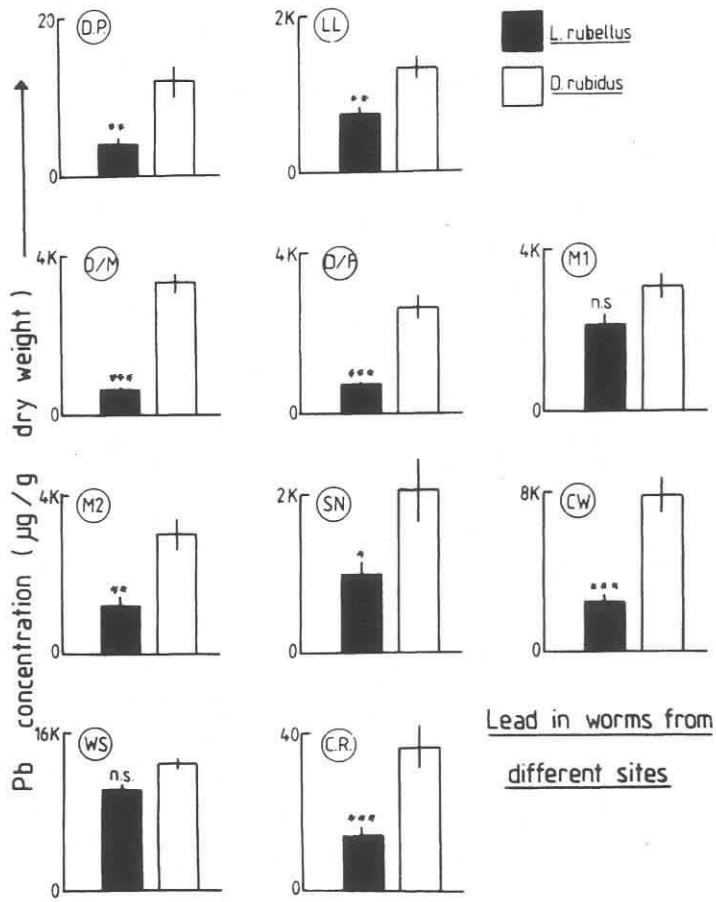


FIGURE 2. Histograms comparing the Pb concentrations in two epigeic earthworms (*L. rubellus* and *D. rubidus*) sampled from an uncontaminated site (D.P.) and nine disused metalliferous sites (the others). Data are presented as means ± S.E.; number of observations vary from 7 to 42. Symbols: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and n.s. = non significant. Capital letters in circles are site codes. (Figures are drawn from data presented in tabular form by Morgan, J. E. and Morgan, A. J., *Bull. Environ. Contam. Toxicol.*, 47, 296, 1991. With permission.)

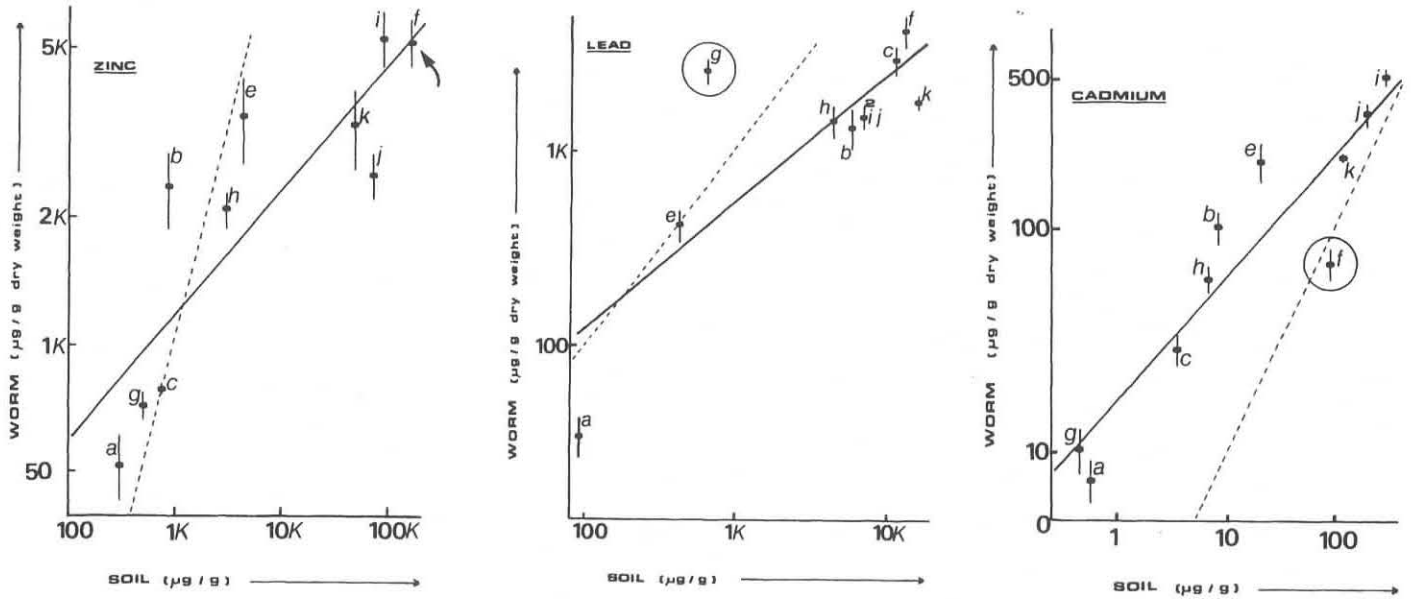
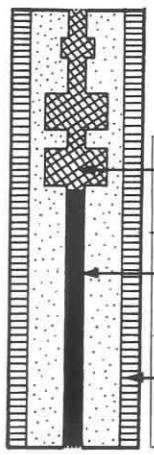


FIGURE 3. Relationships between Pb ($\log_{10} Y = 0.801 + 0.642 \log_{10} X$; $r^2 = 66.5$), Zn ($\log_{10} Y = 2.20 + 0.289 \log_{10} X$; $r^2 = 72.3$), and Cd ($\log_{10} Y = 1.23 + 0.553 \log_{10} X$; $r^2 = 85.6$) concentrations in whole-worm *(L. rubellus)* tissues and the soils that they inhabited. Vertical lines represent standard errors about geometric means; letters (a to l) refer to the different sampling sites; "a" is an uncontaminated site, the others are disused metalliferous sites. Broken lines are unity lines. Circled data points ("g" in the case of Pb; "f" in the case of Cd) are subjectively considered to be outlier populations. Note that the soil at site "f" has the highest Zn concentration. (These figures are redrawn from figures published by Corp, N. and Morgan, A. J., *Environ. Pollut.*, 74, 39, 1991. With permission.)



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	MEAN % DISTRIBUTION IN TISSUE FRACTIONS					
	Dry weight	Cu	Cd	Pb	Zn	Ca
Anterior alimentary fraction (AF)	10.8	12.3	8.8	4.4	10.5	67.0
Posterior alimentary fraction (PF)	22.0	57.3	72.8 (66)	87.7	78.8	21.3
"Rest" fraction (RF)	67.0	30.4	18.4	7.9	10.7	11.7

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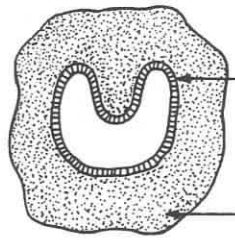

	MEAN % CADMIUM CONTENT OF COMPONENTS OF THE POSTERIOR ALIMENTARY FRACTION	
	L. rubellus	A. caliginosa
Intestinal epithelium (IE)	58.7	66.0
Chloragogenous tissue (CH)	41.3	34.0

FIGURE 4. A. Mean percentage distribution of dry weight, and metal (Cu, Cd, Pb, Zn, Ca) contents (μg) in three major tissue fractions of the earthworm *L. rubellus* sampled from a Pb/Zn mine site at Llantrisant, South Wales. The AF includes the esophagus, calciferous glands, crop, and gizzard; PF includes the intestinal epithelium and chloragogenous tissue; RF consists mainly of the body wall. (The data are derived from Morgan and Morgan;[15] the Cd value in parentheses was estimated from data presented by Ireland and Richards,[42] and was included for comparative purposes.)
B. Mean percentage Cd content in the two major components of the posterior alimentary fraction of two ecophysiologically different earthworm species, *L. rubellus* and *A. caliginosa*, maintained under laboratory conditions on Cd-soaked filter paper. Note that the Cd content of the intestinal epithelium is higher in both species than the Cd content of the chloragog. (Values were calculated from data presented by Ireland, M. P. and Richards, K. S., *Environ. Pollut. Ser. A*, 26, 69, 1981. With permission.)

pathway, for example: (1) it inhibits the enzyme delta-aminolevulinic acid dehydratase (ALAD), leading to the accumulation of delta-aminolevulinic acid; (2) it inhibits the enzyme coproporphyrinogen oxidase, leading to the excretion of coproporphyrin; and (3) in inhibits ferrochelatase, leading to the accumulation of protoporphyrin IX. Hemoglobin is the respiratory pigment of earthworms.[49] Available evidence suggests that the tissue site of haem synthesis in earthworms is the chloragog,[50] the very tissue that accumulates Pb to concentrations as high as >20,000 μg/g dry mass in the intracellular chloragosom granules.[44] Not surprisingly, Ireland and Fischer[51] were able to reduce the activity of the enzyme ALAD in the chloragog tissue of *L. terrestris* maintained on a Pb-spiked filter paper for several days under laboratory conditions. Our own provisional observations on *L. rubellus* sampled from an uncontaminated and two different Pb-contaminated sites indicated that the effect of Pb on haem synthesis under field conditions may be rather more complicated (see Figure 6).

The major conclusions of our analytical study, involving analysis by High Pressure Liquid Chromatography (HPLC) of chloroform extracts of whole *L. rubellus*, can be summarized as follows:

1. The dominant porphyrin in the earthworm tissues was protoporphyrin IX.
2. The concentration of protoporphyrin IX in the tissues of worms inhabiting one contaminated site (Cwmystwyth) was twice as high as in 'control' worms (Dinas Powys).
3. The protoporphyrin IX concentration in the tissues of worms inhabiting a second contaminated site (Llantrisant) was only half that measured in the Dinas Powys 'controls'.
4. The extremely dark, violet-brown pigmentation of the Cwmystwyth worms indicated that the protoporphyrin IX accumulates primarily in the body wall.

It has been shown that Pb accumulation inhibits ALAD activity, while Zn activates the enzyme and protects it from Pb-inhibition.[52,53] Ferrochelatase, the enzyme catalyzing the insertion of ferrous iron into protoporphyrin IX to produce hemoglobin, may be similarly activated by Zn and inhibited by Pb. Our observations suggest that the elevated protoporphyrin levels in Cwmystwyth worms may be due to two causative factors: (1) high tissue Pb concentrations accumulated from the moderately Pb-contaminated soil due to the low pH of the soil matrix,[36] and (2) tissue Zn concentrations that are too low to compensate fully for Pb-inhibition by reactivating ferrochelatase. The lower protoporphyrin IX concentration in the worms inhabiting the relatively heavily Pb- and Zn-contaminated, but neutral soil at Llantrisant may reflect the relatively low Pb and high Zn tissue concentrations in this population.

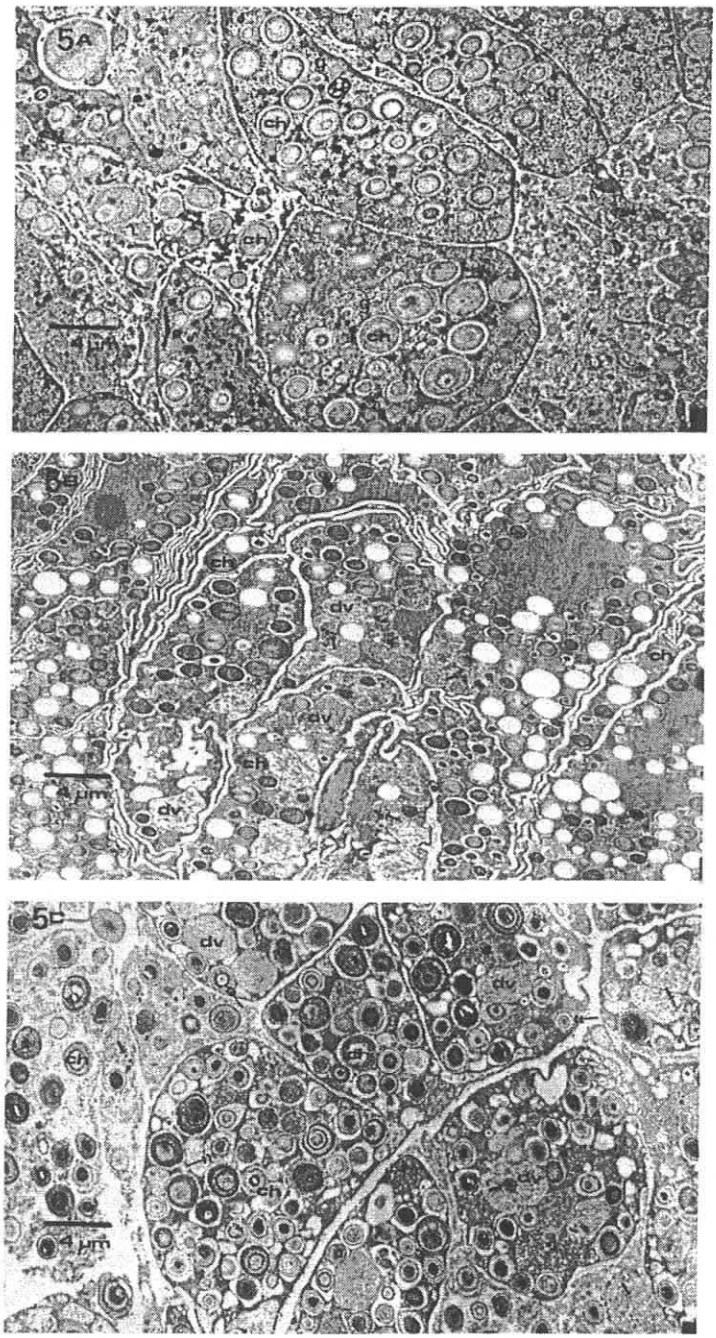
5A
ch
4 µm
5B
dv
5C

FIGURE 5. Transmission electron micrographs (low magnification) providing an overview of the morphology of the chloragocytes of *L. rubellus* inhabiting: an uncontaminated site (5A: Dinas Powys); an acidic site with relatively low total, but high 'available' soil Pb concentration (5B: Cwmystwyth, site "g" in Figure 3; see also Figure 6); a calcareous site heavily contaminated with Pb, Zn, and Cd; (5C: Draethen; see Figure 7). Note that there are a number of obvious differences between the 'normal' and the 'stressed' cells. Apart from the characteristic chloragosome granules (ch), the control chloragocytes contain vast quantities of glycogen (g). The stressed cells contain very little glycogen; they do, however, contain large numbers of debris vesicles (dv) packed between the chloragosomes. The debris vesicles often contain fine dense particulates (arrows) dispersed within more lucent material; occasionally, the chloragosomes also contain small dense particles, especially near their peripheries. (The origins and fate of these structures cannot be established merely from micrographs such as these; in addition, the appearance of some of the structures, including the distribution of the microparticulates, may be the result of the leaching and redistribution of materials during chemical fixation, dehydration, and resin embedding.)

IV. SUBCELLULAR COMPARTMENTATION OF METALS WITHIN EARTHWORM CHLORAGOCYTES

Three main intracellular pathways have evolved for the binding and immobilization of metals accumulated in terrestrial invertebrates.[54-56] Two of these pathways coexist in earthworm chloragocytes (Figure 7).

The type A pathway is exemplified by phosphate-rich chloragosome granules that bind Ca and Zn.[3,21,43,57,58] It should be noted, however, that the multifunctional chloragosomes differ from the more inorganic phosphate-rich calcospherites that typically occur in most other invertebrates. Earthworm chloragosomes contain a complex mixture of organic macromolecules,[59] and they may have the capacity to release ions for buffering the coelomic fluid and blood.[3,60] Lead appears to bind preferentially to the matrix of chloragosomes (Figure 7), with some evidence suggesting that it does so by effectively displacing Ca^{2+} ions.[44,61] Given the high concentration of Pb that can be bound by the chloragosomes of worms inhabiting heavily polluted soils,[44] it is important to evaluate the efficiency of Pb transport, binding and extrusion mechanisms, and their relationship to cellular Ca status. Lead ions can, as we saw above, inhibit enzymes involved in haem synthesis. Lead may also interfere with cellular function by disturbing Ca homeostasis in one of two general ways. First, it has been claimed,[62] but subsequently challenged,[63] that picomolar concentrations of Pb can mimic Ca in the activation of protein kinase C, an enzyme intimately involved in cellular growth and differentiation. Second, by either binding to SH-containing enzymes such as Ca^{2+} transport ATPases,[64] or by displacing Ca from

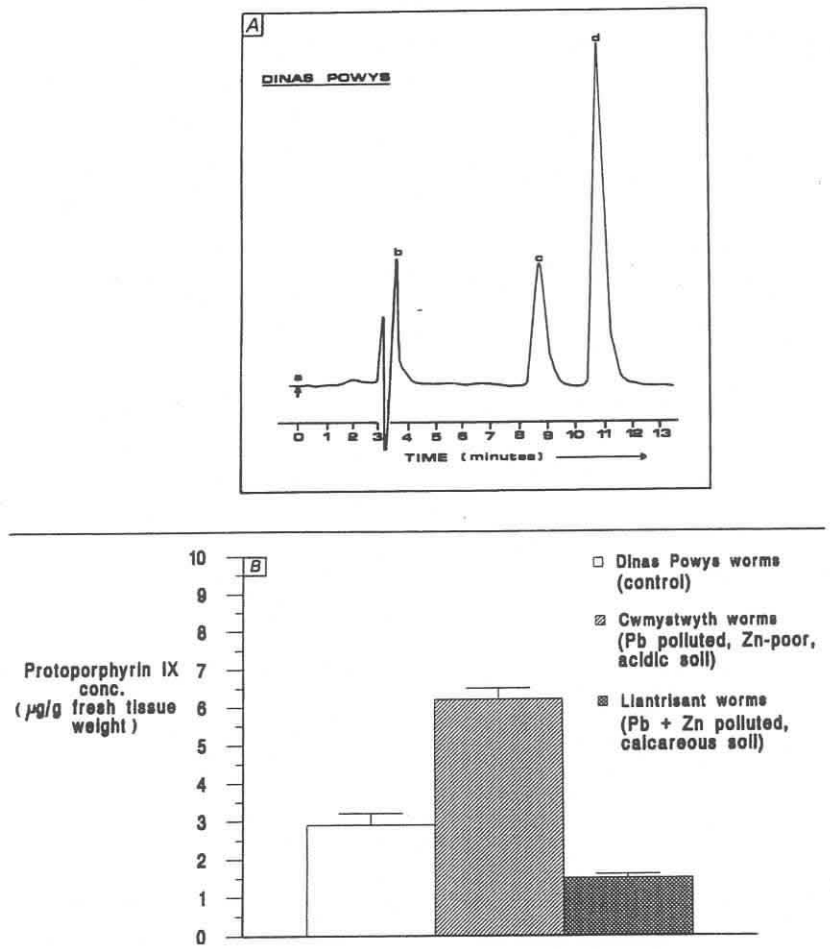

FIGURE 6. The effects of Pb and Zn on haem biosynthesis in *L. rubellus* sampled from an uncontaminated site (Dinas Powys) and two contaminated sites, one (Cwmystwyth) with an acidic soil containing a high proportion of bioavailable Pb but low Zn, and the other (Llantrisant) with a calcareous soil containing high absolute concentrations of both Pb and Zn. After a 3-day period on filter paper to clear their guts, individual worms were weighed, frozen in liquid N_2, thawed, and homogenized. The homogenates were then extracted in 5% H_2SO_4 in methanol, and the porphyrin methyl esters were extracted into chloroform. Porphyrin analysis was performed in a Gilson HPLC, using a Hypersil silica-packed column (25 cm × 4.6 cm i.d.) and a 100 μl injection loop; the wavelength was set at 400 nm, and the solvent system used was 20% ethyl acetate and 80% hexane. (To check the identity of the worm porphyrin, standard protoporphyrin IX, mesoporphyrin IX, and deuteroporphyrin IX were also injected under identical analytical conditions.)

intracellular binding sites, Pb may cause a rise in the concentration of cytosolic free Ca^{2+} leading to a loss of cellular viability due to the activation of phospholipases and endonucleases (see Nicotera et al.[65]). It is not known whether any of these cytotoxic effects are manifest in earthworm chloragocytes exposed to Pb, or whether the populations of earthworms inhabiting polluted sites have evolved mechanisms that safeguard against Pb toxicity or confer resistance to Pb toxicity. An example of an effective resistance mechanism would be the induction of the synthesis of more chloragosomal material by the displaced Ca, so that the capacity to bind Ca (and Pb) is maintained, thus reducing the chance of Ca overload within sensitive organelles.

The type B pathway is characterized by the ability of sulfur-donating ligands to bind Cd. Low molecular weight Cd-binding proteins, exhibiting many of the characteristics of metallothioneins, have been identified in earthworms experimentally exposed to Cd or exposed to the metal in their native soils.[7,66,67] Electron microprobe studies suggest, but cannot confirm, that the Cd-metallothionein complex is confined within vacuoles (Figure 7) possibly of lysosomal origin. A number of authors have in fact suggested that toxic metals bound to metallothioneins are compartmentalized within elements of the lysosomal systems of certain terrestrial and aquatic invertebrates.[68-76] In some cases the accumulation of Cd, and the expression of Cd-resistance, has been shown,[77,78] or suggested,[79,80] to be determined by duplications of metallothionein genes.

Because our knowledge of the mechanisms of metal binding within the earthworm chloragocyte is so relatively poor, it is proposed to highlight certain aspects of the data presented in Figure 7 hopefully, to, stimulate further research on this cell and other metal-accumulating cell types.

The X-ray maps of freeze-dried, cryosectioned, chloragocytes from *L. rubellus* inhabiting a Pb, Zn, and Cd-contaminated mine site indicate that Pb, Zn, and Ca are preferentially bound to the Pb-rich matrix of chloragosomes, and that Cd is confined to S-rich compartments called, perhaps misleadingly, ''cadmosomes''.[27,81] X-ray microanalysis is an analytical tool of high spatial resolution, but poor sensitivity. The minimum detectable 'local' concentration of an element varies with its atomic number, but even under the most favorable conditions, is not better than about 200 μg/g dry mass.[82] Thus it is possible that the chloragosomes could contain significant, albeit nondetectable, amounts of Cd, and the cadmosomes significant amounts of Pb and Zn. If this were the case, the metal accumulating pathways in the chloragocyte may not be as discriminatory as the maps suggest. Earlier 'static' microprobe analysis of the chloragocytes of Draethen worms indicated that at least three distinct metal-

A: A typical analytical spectrum of porphyrins from Dinas Powys ('control') worms, a = sample injection point; b = solvent front; c = unidentified peak at 8 min; d = major protoporphyrin IX peak at 11 min.
B: Histogram (means ± S.D.) comparing the concentration of protoporphyrin IX in whole earthworms from the three study sites.

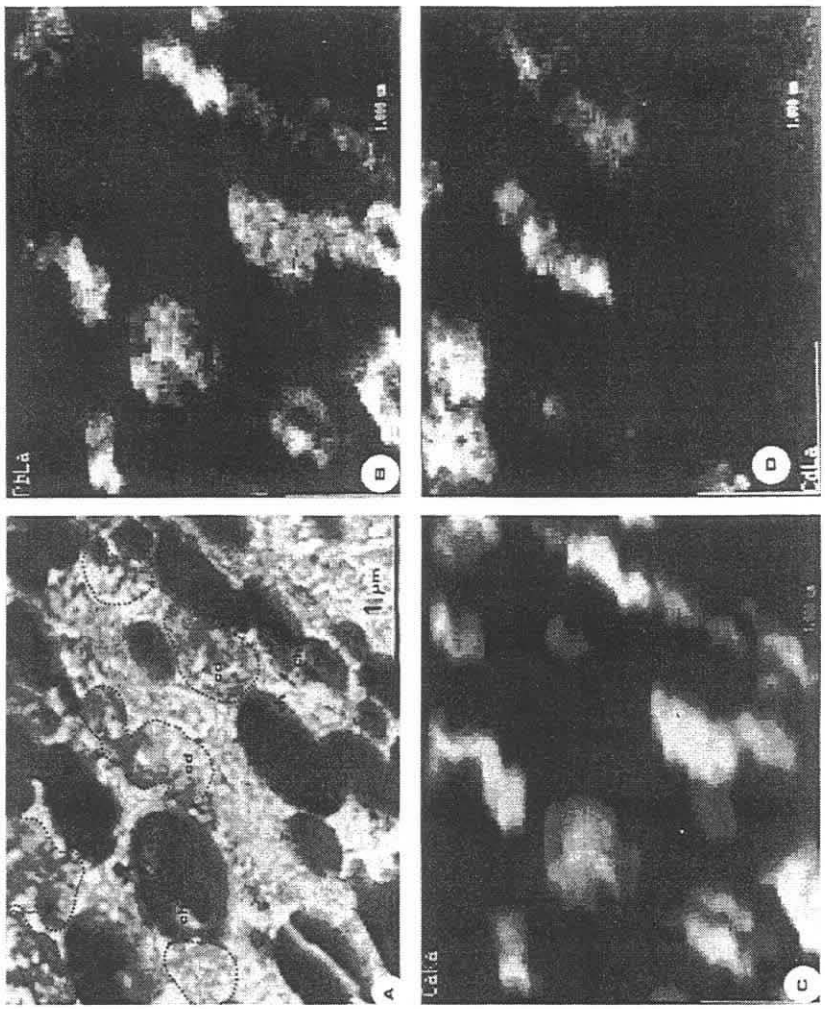

FIGURE 7 A-D.

FIGURE 7. A: Scanning transmission electron micrographs (bright field image) of a freeze-dried, unfixed, thin cryosection of a chloragocyte from an earthworm, *L. rubellus*, sampled from a site (Draethen) contaminated with Pb, Zn, and Cd. The fresh tissue was mounted on a metal pin and plunged into stirred liquid propane, sectioned on a glass knife at −120°C in a Reichert Ultracut E fitted with an FC4 cryochamber, and the thin (~100 nm) sections mounted on coated titanium grids and transferred in a liquid N_2-cooled aluminum lidded pot to a vacuum coating unit, where they were freeze dried and coated with carbon. The electron opaque chloragosome granules (ch) are prominent in the micrographs (compare with Figure 5); the cadmosomes (cd) are more difficult to discern morphologically and have been delineated with dotted lines after reference to the Cd X-ray map (Figure 7D).

accumulating compartments could be compositionally, if not morphologically, identified.[61] These were the typical chloragosomes and cadmosomes, and a 'hybrid' compartment containing Pb, Zn, and Cd. One interpretation of these observations is that the chloragosomes and cadmosomes are distinct lysosomal lineages that 'converge' to form a tertiary lysosome, or residual body, which may then be disposed of in a yet unknown manner. This hypothesis, derived in part from that suggested by George[83,84] to describe the compartmentation of Cd in *Mytilus* kidney, requires further testing.

Metallothioneins and similar proteins have long been recognized to play important roles in the intracellular homeostasis of essential metals, such as Zn and Cu, by perhaps storing and donating these metals to meet metabolic needs.[85] Goering and Fowler[86] demonstrated *in vitro* that Zn-metallothionein could donate Zn to, and thus activate, the enzyme delta-aminolevulinic acid dehydratase. More recently, Sunderman[87] showed that Cd could substitute for Zn in the DNA-binding ''zinc finger'' proteins that regulate gene expression (see Klug and Rhodes[88] and Evans and Hollenberg[89]). It is therefore possible that not only are the Zn-dependent transcription factors the molecular targets for Cd toxicity (it is of more than passing interest that Cd is a potent genotoxin in the Zn-rich reproductive tissues of mammals), but that metallothioneins may have the capability of transporting and donating Cd to the vulnerable intracellular targets. On the other hand, the confinement of Cd-metallothionein complexes within vesicular structures in at least some invertebrate cells may be a mechanism of harnessing the capacity of metallothionein to sequester toxic metals without the freedom to transport and redistribute them freely within the cell. Indeed, George[83] showed that the tertiary lysosomes in the kidney cells of *Mytilus* contained Cd but no metallothionein, suggesting that the protein had been hydrolyzed, and perhaps that the released amino acids had been recycled for further synthetic purposes.

Copper causes mortality and sublethal toxic injury to earthworms at environmental concentrations much lower than do Pb and Zn.[90] Furthermore, earthworms do not appear to accumulate high Cu concentrations even from heavily

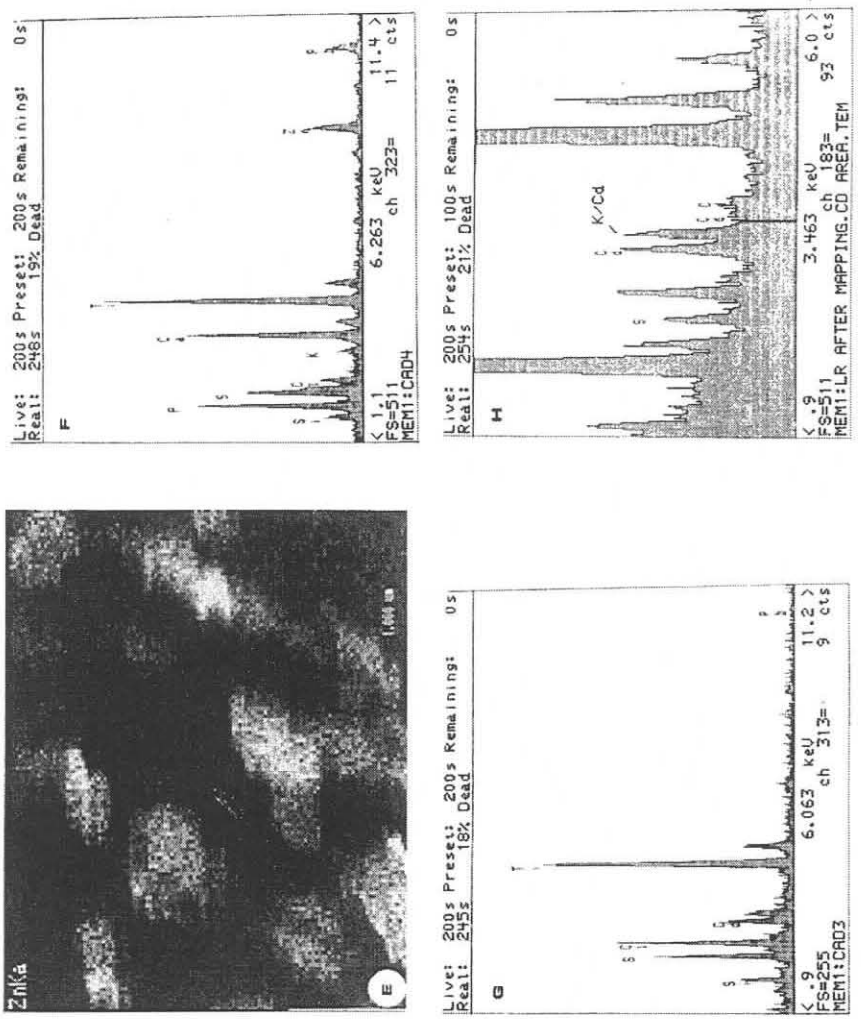
ZnKa
E
Live: 200s Preset: 200s Remaining: 0s
Real: 248s 19% Dead
F
6.263 keV
ch 323=
11 cts
FS=511
MEM1:CAD4
Live: 200s Preset: 100s Remaining: 0s
Real: 254s 21% Dead
H
K/Cd
3.463 keV
ch 183=
93 cts
MEM1:LR AFTER MAPPING.CD AREA.TEM
Live: 200s Preset: 200s Remaining: 0s
Real: 245s 18% Dead
G
6.063 keV
ch 313=
9 cts
FS=255
MEM1:CAD3

FIGURE 7 E-H.

FIGURE 7. B to E: Electron generated X-ray maps for Pb (B), Ca (C), Cd (D), and Zn (E) in the area depicted in the micrograph. Note that the Pb, Ca, and Zn are codistributed in the chloragosomes and that Cd is equally discretely distributed but is confined to a second metal-sequestering compartment, the cadmosome. In this cellular field, there appears to be no overlap in the distributions of Pb and Cd — this was more convincingly apparent in the colored maps from which these figures were derived. With color it is possible to superimpose maps for two or more elements so that their relative distributions can be appreciated readily at high spatial resolution. However, the specificity of intracellular compartmentation is confirmed by the X-ray spectra, obtained with 'stationary probes' positioned on a chloragosome (F) and a cadmosome (G and H), respectively. Note the presence of Zn in spectrum F, but not in G. Conditions for mapping and/or spectral acquisition were JEOL JEM-2000 FXII STEM operated at 120 kV, with 12 microamps heating current on a LaB_6 emitter. Spectra were acquired with 200 s 'live' time using a horizontal Link Pentafer ultra-thin-window detector fitted with a 30 mm^2 silicon crystal (FWHM resolution = 133 eV at 5.9 keV); Link Systems eXL X-ray analyzer; screen resolution = 128 × 128 pixels; dwell time on each pixel = 5 s; total map acquisition time = $22^3/_4$ h; condenser aperture = 200 μm. Background was subtracted from all spectra to exclude the effects of local specimen topographical effects on the maps; most maps (all except Zn) were median filtered to eliminate 'noise' (single random black or white points), but test points were always made without filtering to ensure that no data were lost or artificially created by this process. Drift correction was not available on the equipment used for mapping, but this was not a serious handicap because no significant drift was observed even though we analyzed a thin cryosection for a protracted period. Micrographs of the analyzed areas were taken immediately before and after analysis, either directly from the STEM unit onto Polaroid film or via the X-ray microanalyzer. Colored X-ray maps were recorded using a Mitsubishi CP200B color printer connected to the video output of the analyzer.

contaminated soils.[14,15] The high toxicity and low accumulation of Cu may be a function of the inability to induce the metallothionein gene in earthworms, despite the fact that Cu probably would form a more stable complex with the protein than does Cd (see Bremner[91]). Thus, we hypothesize that earthworms inhabiting a Cd-contaminated soil, and whose metallothionein gene(s) are activated, would not only be more Cu resistant, but would accumulate relatively high Cu concentrations in the so-called cadmosome vesicle.

There is no doubt that transintestinal transport is a major mode of entry of metals into earthworm tissues,[4] although transintegumentary uptake may not be insignificant. In earthworms, unlike terrestrial macroinvertebrates with relatively inpermeable exoskeletons, such as isopods and spiders,[56] the metal storage mech-

anism of the alimentary epithelium has been largely neglected. Ireland and Richards[42] (see Figure 4) showed that the intestine accumulated high Cd concentrations in worms exposed to filter paper soaked with a 5 ppm solution of Cd for 26 days. This suggests that the epithelium acts as a major barrier limiting the entry of Cd into the body fluids. The relationship between the Cd burden of the intestine and that of the chloragog has not been studied. Does the chloragog merely accumulate Cd that cannot be sequestered by the intestine? If so, it would be expected that the proportion of Cd bound by the intestine relative to the chloragog would decrease as the body burden increased. Are metallothionein gene(s) expressed in the intestinal cells? If so, is the Cd-metallothionein complex confined to "cadmosomes", or other components of the lysosomal system? Is metallothionein implicated in the transfer of Cd from the intestinal cells to the chloragog? Morgan et al.[67] showed that the posterior alimentary tissue fraction of *L. rubellus* contained two slightly different Cd-binding metallothionein-like proteins. Is one confined to the chloragog and the other to the intestine, or do they coexist in both tissues? Does Cd accumulation alter the structure and function of the intestinal epithelium? It is less likely that the integumentary cells act as metal storage and transit sites, because a very high proportion of the body wall Cd burden appears to be associated with surface mucus and not internalized.[42]

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